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in Breast Cancer Cells

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13. ABSTRACT (Maximum 200 Words) The purpose of this grant is to understand how activation of the NF- κ B/Rel family of transcription factors leads to breast cancer cell survival following treatment with radiation. The NF- κ B/Rel family of transcription factors are known to greatly affect survival of various cancer cell types, including breast cancer cells. Our hypothesis is that activation of NF- κ B in breast cancer cells contributes to a G ₂ /M cell cycle arrest, affording these cells extra opportunity to repair damaged DNA and thus allowing them to evade death inducing effects of radiation. Cell cycle analysis and levels of apoptosis were determined following exposure to ionizing irradiation. Cells capable of NF- κ B activation efficiently arrested in G ₂ /M cell cycle phase while those that are not capable of activating NF- κ B, do not efficiently arrest. Using RPA analysis we identified a gene, p21 ^{waf1/cip1} , and have now shown that it is involved in maintaining the G ₂ /M phase cell cycle arrest following IR. Through the use of stable RNAi interference, we found that the G ₂ /M arrest is partially dependent on p21 ^{waf1/cip1} . Understanding how NF- κ B is activated and how NF- κ B provides protection from cell death will be important for designing strategies to circumvent this resistance mechanism to improve efficacy of radiation therapy.				
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Annual Summary Report

I. Introduction

Background: The nuclear factor κ B (NF- κ B) family of transcription factors regulates expression of genes critical for multiple biological processes, including immune responses, inflammatory reactions, cell proliferation, cell differentiation, and apoptosis. Traditionally, activation of NF- κ B involves an extracellular signal that disassociates the inhibitor protein I κ B α from NF- κ B, allowing its translocation to the nucleus. Recently, NF- κ B has been shown to be activated by DNA damaging agents which cause DNA double-strand breaks (DSB) in several tumor cell lines, including breast cancer lines. These DNA damaging agents are the same chemotherapeutic agents used in the treatment of breast cancer. NF- κ B activation has been linked to both cell survival and cell death by inducing the expression of anti-apoptotic genes and apoptotic genes, depending on cell types and inducing agents. Surprisingly, studies in our laboratory further demonstrated that there are many cancer cell lines that fail to activate NF- κ B by DSB-inducing agents, indicating that this activation pathway is not a universal phenotype of all cancer cell types. In addition, many normal human cell types fail to activate NF- κ B by several DSB inducing agents. Thus, our studies suggest that NF- κ B activation by certain DSB-inducing agents may be an "acquired" phenotype of certain malignant cells.

Objective/hypothesis: The purpose of this proposal is to further understand the mechanisms of NF- κ B related G₂/M cell cycle arrest. We believe NF- κ B can be activated by DSB-inducing agents and that this activation leads to protection from apoptosis by initiating a cell cycle arrest, allowing more opportunity to repair damaged DNA and resistance to anti-cancer agents.

II. Body

(Submitted paper to Molecular Cancer Research)

Enhanced cancer cell survival by NF- κ B-dependent p21^{waf1/cip1} induction.

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ABSTRACT

Nuclear factor kappaB (NF- κ B) regulates cell survival pathways, but the molecular mechanisms involved are not fully understood. Here, we developed an NF- κ B-reporter T leukemic cell system to monitor the consequences of NF- κ B activation by DNA damage insults. Cells that had activated NF- κ B following treatment with ionizing radiation or etoposide arrested in the G₂/M phase for prolonged time, which was followed by increased cell cycle re-entry. In contrast, those that had failed to activate NF- κ B underwent transient G₂/M arrest and extensive cell death. NF- κ B-dependent induction of the cyclin-dependent kinase inhibitor p21^{waf1/cip1} in S-G₂/M phases contributed to the maintenance of this cell cycle arrest while RNA interference of p21 reduced the arrest. Thus, p21^{waf1/cip1}-dependent G₂/M arrest protects cells from apoptosis and represents an NF- κ B-dependent cancer resistance mechanism.

INTRODUCTION

The Rel/NF- κ B family of transcription factors regulates expression of genes critical for multiple biological processes, including immune responses, inflammatory reactions, and apoptosis (1, 2). In particular, many recent studies underscore the importance of NF- κ B in the induction of genes involved in resistance to chemotherapeutic agents and radiation therapy (reviewed in (3)). These genes include antiapoptotic Bcl-2 family members and the cIAP family of caspase inhibitors, among others (4-8). However, the repertoire of NF- κ B regulated genes that participate in cell survival regulation is incompletely understood.

Inactive NF- κ B complexes exist in the cytoplasm in association with inhibitors, such as I κ B α , and release from these inhibitors is critical for NF- κ B to enter the nucleus and activate gene expression (1). Many signaling cascades that control NF- κ B activation converge on an I κ B kinase (IKK) complex that is responsible for releasing NF- κ B. Phosphorylation of I κ B α on serines 32 and 36 leads to its ubiquitination and subsequent degradation by the 26 S proteasome (9). Because of the sequential nature of this signaling pathway, there are many steps in which the activation of NF- κ B can be inhibited. One method is to express a mutant form of I κ B α (S32/36A-I κ B α or so called super-repressor I κ B α mutant) that harbors mutations at the IKK phosphorylation sites and consequently is not targeted for the degradation pathway to liberate NF- κ B. Alternatively, while not as selective for the pathway as the above I κ B α mutant, proteasome inhibitors have also been employed to prevent the NF- κ B pathways (10-13). Combined with knock-out studies of the NF- κ B family and IKK components, these "loss-of function" approaches were instrumental in determining the role of NF- κ B as a key survival factor in both physiological and pathological settings (14-17). These approaches also helped to define NF- κ B regulated genes whose expression was lost upon inhibition of NF- κ B activation, thus, correlating with its antiapoptotic activities.

However, the role of NF- κ B in cell death regulation varies greatly depending on the cellular contexts and the stress signals utilized (18, 19). This disparity is presumably due to the differences in regulation of NF- κ B target genes under different

experimental settings. These discrepancies may, in part, stem from differences both in the percentage of a cell population among drug-exposed cells that is capable of activating NF- κ B and the magnitude of activation within each stressed cell, both of which may vary greatly under different conditions. For example, when cells are treated with topoisomerase I inhibitors, such as camptothecin (CPT) and its derivatives, only cells in the S-phase seem to activate NF- κ B (20, 21). Thus, any NF- κ B-dependent effects will be initiated primarily from this cell population. Variations in the percentage of S-phase populations in drug-exposed cells will then introduce variations in NF- κ B-dependent phenotypes. In other conditions, such as anticancer DNA damaging agents, including ionizing radiation (IR) and the topoisomerase II inhibitor etoposide (VP16), it is unknown whether NF- κ B activation is regulated in a cell cycle-dependent manner or other undefined cellular contexts. Without a means to trace live NF- κ B-activated cell populations, it is difficult to examine the populational variation of NF- κ B activation and the consequences within those NF- κ B-activated cells. Thus, there is a need to develop a cell-based NF- κ B reporter system to evaluate the behavior of these distinct cell populations to directly link NF- κ B activation to specific cell survival gene regulation and phenotypes.

Tumors with a defective tumor suppressor p53 occur frequently in different types of cancer and are generally more resistant to chemotherapy (22-25). Cells in which p53 is inactivated tend to have a selective growth advantage partly due to the lack of target genes normally controlled by p53. These genes are involved in various cellular activities, such as growth arrest, induction of apoptosis, inhibition of angiogenesis, among others (reviewed in (26)). For example, following genotoxic stress challenges, p53 controls transcription of numerous proapoptotic members of the Bcl-2 family, such as Bax, Puma, Noxa, and Bid (reviewed in (22)). P53 also regulates cell cycle arrest by transcriptional activation of p21^{waf1/cip1}, a member of the Cip/Kip family of cyclin dependent kinase inhibitors that mediates the p53-induced G₁ growth arrest (27, 28). Due to loss of p21^{waf1/cip1} regulation, p53-deficient cells often display a lack of a G₁ cell cycle arrest following treatment with anticancer drugs (29-31). Because of the presence of other checkpoint mechanisms mediated by the activation of different kinases depending on the type of DNA lesions, p53-deficient cells still possess cell cycle checkpoint mechanisms in other cell cycle phases (32-34). However, mechanistic linkages between cancer resistance and NF- κ B activation resulting from p53-deficiency are not well established.

To address cancer resistance mechanisms in relation to cell cycle regulation by NF- κ B activation, we developed a "positive selection" strategy by engineering an NF- κ B reporter cell system using a p53-mutant CEM T leukemic cell line. This system revealed a previously unrecognized G₂/M maintenance role for NF- κ B through the induction of p21^{waf1/cip1}. Additionally, p53-mutant breast cancer cells also induced p21^{waf1/cip1} in an NF- κ B dependent manner and arrested in G₂/M for prolonged time, leading to their increased survival. Thus, the NF- κ B- p21^{waf1/cip1}-G₂/M maintenance pathway elucidated here may be a contributor to the development of resistance in certain p53-defective human cancer types.

EXPERIMENTAL PROCEDURES

Cell culture and Generation of CEM_κB cells. CEM human T leukemic and MDA-MB-231 human breast cancer cell lines, along with their derivatives, were maintained in RPMI 1640 medium (Mediatech; Herndon, Virginia) supplemented with 10% fetal bovine serum (HyClone Laboratory, Inc; Logan, Utah), 100 units of penicillin G and 100 μg/ml streptomycin sulfate (Mediatech; Herndon, Virginia). The 3x_κB-GFP reporter was constructed by removing the CMV promoter from the pEGFP-C1 plasmid (Clontech) and replacing it with the 3x_κB-tk promoter from the 3x_κB-LUC plasmid (20). The 3x_κB-GFP was introduced into CEM by electroporation as described below and selected with 1 mg/ml G418 (Mediatech; Herndon, Virginia). Cells were stimulated with TNF α and those that expressed GFP were positively selected by FACS sorting as described below. These cells were then incubated in the absence of TNF α to ensure that the GFP levels dropped to undetectable levels. This process was repeated two more times to isolate a clone of CEM_κB cells that possessed the capacity to respond to TNF α stimulation robustly. This process ensured the isolation of cell clone that contained the 3x_κB-GFP reporter gene stably integrated into a genomic location(s) that efficiently responded to NF- κ B stimulation. Expression of the super-repressor I κ B α showed the specificity of NF- κ B-dependent GFP induction in the CEM_κB clone (data not shown).

Reagents and antibodies. A JL Shepherd Model JL-109 with a ¹³⁷Cs source was used for γ -irradiation. Camptothecin (CPT), doxorubicin (Dox), etoposide (VP16), and propidium iodide (PI) were purchased from Sigma (St. Louis, Missouri). Human recombinant TNF α was purchased from Calbiochem (La Jolla, California). Actin (C-11), I κ B α (C-21), p21 (F-5), p21 (C-19) and p53 (D0-1) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, California). Anti-rabbit and anti-mouse antibodies conjugated to horseradish peroxidase were obtained from Amersham Pharmacia Biotech (Piscataway, New Jersey) while anti-goat antibody conjugated to horseradish peroxidase was obtained from Santa Cruz Biotechnology.

Western immunoblot analysis, electrophoretic mobility shift assay (EMSA) and immunoprecipitation. Cell preparation and Western blotting were performed as described (35). The Ig κ - κ B and Oct-1 probes and conditions for EMSA were as described (36). To detect p21^{waf1/cip1} protein in CEM_κB cells, 5 x 10⁶ cells were lysed in 10% PBS and 90% lysis buffer as described previously (Huang 2002, MCB). Supernatants were diluted further in lysis buffer, and 1 μg of p21 antibody (C19, Santa Cruz) was added to each tube. Samples were rotated for 60 min at 4°C. Protein G-Sepharose beads (Amersham Pharmacia Biotech; Piscataway, New Jersey) were then added to each tube, and the samples were rotated overnight at 4°C. P21^{waf1/cip1} protein was resolved in 12% SDS-PAGE gels and analyzed by Western blotting using the same antibody.

FACS sorting, cell cycle analysis, and fluorescent microscopy. CEM_κB cells were exposed to 10 Gy of IR and allowed to repair for a total of six hours at 37°C. "GFP-positive" CEM_κB cells were sorted from "GFP-negative" cells using the FACS Vantage cell sorter (BD Pharmingen; San Jose, California) followed by immediate fixation in 70% ETOH. For cell cycle analysis, cells were processed as previously described (37) and analyzed on a FACScan flow cytometer (BD Pharmingen; San

Jose, California). Data were analyzed using the Cellquest (BD Pharmingen; San Jose, California) and ModFit (Verity Software House; Topsham, Maine) software. CEM_kB cells were visualized and photographed as described previously (38).

RNAse Protection Assays (RPA). CEM_kB and CEM-S32/36A cells were irradiated at a dose of 20 Gy. Following a 5.5 hour incubation, cells were labeled for 30 min. with Hoechst 33342 dye (Molecular Probes; Eugene, Oregon) before sorting on the FACS Vantage cell sorter. Cells in the G₁ phase of the cell cycle were sorted from the total of the population. Cells were collected at 4°C, washed in PBS, and immediately froze at -70°C. Cells were homogenized using a Qias shredder column (Qiagen; Valencia, California) followed by RNA isolation using the RNeasy kit (Qiagen; Valencia, California). The RiboQuant RPA kit (BD Pharmingen; San Jose, California) was used to perform ribonuclease protection assays on the isolated RNA by hybridizing it with the hStress-1 multi-probe template set (BD Pharmingen; San Jose, California) as outlined in the manufacturer's directions.

Quantitative RT-PCR Analysis. Total RNA from CEM cells treated with 10 μM VP16 for 6 hours was extracted using the RNeasy kit and Qias shredder (Qiagen; Valencia, California). cDNA was synthesized as described (39). Quantitative real time RT-PCR reactions (25 μl) contained 2 μl of cDNA, 12.5 μl of SYBR Green (Applied Biosystems, Foster City, California), and the appropriate primers. Product accumulation was monitored by SYBR Green fluorescence. The relative expression levels were determined from a standard curve of serial dilutions of cDNA samples. Forward and reverse primers for real time PCR were: (1) human p21^{waf1/cip1}, 5'-GCAGACCAGCATGACAGATTTC-3' and 5'-GCGGATTAGGGCTTCCTCTT-3'; human Bcl-xl, 5'-TGCCTAAGGCGGATTTGAAT-3' and 5'-ATTGTCCAAAACACCTGCTCACT-3'; and GAPDH primers: 5'-GAAGGTCGGAGTCAACGGATTT-3' and 5'-GAATTTGCCATGGGTGGAAT-3'.

Generation of stable p21^{waf1/cip1} pSilencer knockdown clones. P21^{waf1/cip1} RNA interference stable CEM and MDA clones were generated using the pSilencer vector (Ambion; Austin, Texas). A 19-nucleotide RNA interference sequence was chosen to knockdown p21^{waf1/cip1} that had no significant homology to any other gene in the human genome. Two DNA oligos were designed for knocking down p21^{waf1/cip1} following the manufacturer's protocol linking the 19-nucleotide sense and antisense sequences as follows: 5'-CTTCGACTTTGTCACCGAGTTCAAGAGACTCGGTGACAAAGTCGAAGT TTTT-3' (sense) and 5'-AATTA AAAA ACTTCGACTTTGTCACCGAGTCTCTTGAAC TCGGTGACAAAGTCGAAGGGCC-3' (antisense). The scramble control was made as follows: 5'-TACCGTCTCCACTTGATCGTTCAAGAGACGATCAAGTGGAGACGGTA TTTTT-3' (sense) and 5'-AATTA AAAA ATACCGTCTCCACTTGATCGTCTCTTGAAC GATCAAGTGGAGACGGTAGGCC-3' (antisense). The oligos were annealed, and the resulting insert was ligated into the pSilencer vector that had been linearized with the restriction enzymes Apal and EcoRI. The resulting plasmid (40 μg) and a puromycin resistance vector pLPL-CA (4 μg) were cotransfected into both CEM and MDA cells by electroporation at a setting of 300 volts and 950 μF in a Bio-Rad Gene Pulser apparatus with capacitance extender. Stable clones were selected by 1 μg/ml puromycin and screened for their ability to knockdown p21^{waf1/cip1} protein expression by Western blotting.

RESULTS

NF- κ B activation by genotoxic agents in CEM cells. Prior to developing our cell-based NF- κ B reporter system, we determined the time course and dose-response of NF- κ B activation with CEM cells using distinct DNA damaging agents. Cells treated with IR induced dose-dependent NF- κ B activation that was saturated at approximately 10 Gy (Figure 1A) and transient activation that peaked around 3-4 hours (Figure 1B). Supershift experiments showed that the NF- κ B complexes were principally composed of p50 and p65 NF- κ B subunits (not shown). Consistent with previous studies (40, 41), this activation did not require de novo protein synthesis and was associated with degradation of I κ B α protein by 30 min, which was then followed by its resynthesis around 2 hours (Figure 1B, others not shown). Treatment with DNA topoisomerase I or II inhibitors, such as CPT, topotecan (TPT), doxorubicin (Dox), and VP16, also resulted in increased NF- κ B DNA binding activity (Figure 1C) in a dose-dependent and similar transient activation manner (data not shown). Increased transcription of an NF- κ B-dependent luciferase reporter gene (Figure 1D) confirmed that NF- κ B released by these DNA damaging agents in CEM cells was transcriptionally competent.

NF- κ B activation by certain DNA damaging agents occurs in different cell cycle phases. To begin to monitor NF- κ B functions at the single cell level, we developed CEM κ B, a CEM cell-based NF- κ B reporter assay, by stably integrating the 3 κ B-GFP reporter construct in a CEM leukemic T cell line. Multiple rounds of FACS sorting were used to isolate cell clones that displayed low basal GFP fluorescence with high inducible GFP fluorescence following treatment with TNF α (Figures 2A, middle panel and 2B, upper panel, others not shown). This process ensured the isolation of cells that had stably integrated the κ B-GFP reporter at inducible genomic loci. To ensure that the GFP(+) cells did indeed activate NF- κ B and that the GFP(-) cells did not activate NF- κ B, the two populations were analyzed for NF- κ B activity by EMSA following TNF α treatment. The GFP(+) cells displayed NF- κ B binding activity following TNF α treatment while GFP(-) cells displayed relatively little NF- κ B binding activity (Figure 2C). Specific NF- κ B-dependent induction of GFP was confirmed by its inhibition following expression of the super-repressor I κ B α mutant protein (not shown). Interestingly, the total number of GFP(+) cells after treatment with CPT was much less than those treated with TNF α (Figures 2A, lower panels and 2B, lower panels). Moreover, cell sorting for "GFP(+)" and "GFP(-)" populations followed by PI staining showed that the GFP(+) populations following treatment with CPT were greatly enriched for only the S-phase cells, unlike TNF α -stimulated cells that showed GFP induction in different cell cycle phases (Figure 2D). These results in live cells demonstrated that NF- κ B activation by CPT was largely coupled to the S-phase, an observation consistent with previous biochemical studies (20, 21).

Using this assay, we wanted to determine whether other DNA damaging agents activated NF- κ B in specific phases of the cell cycle. In contrast to activation by CPT, which was efficiently prevented by the treatment with the DNA polymerase inhibitor aphidicolin (Figure 2F, lane 4), NF- κ B activation by none of the other DNA damaging agents was sensitive to inhibition by this inhibitor. Thus, S-phase progression was not necessary to cause NF- κ B activation by these other DNA damaging agents. To directly

determine whether NF- κ B activation by IR and VP16 was also cell cycle-dependent, CEM κ B cells were sorted for GFP(+) populations at 6 hours following treatment with these agents. Like TNF α , but unlike CPT (Figure 2D), the GFP(+) populations after VP16 or IR exposure represented DNA contents representing each phase of the cell cycle (Figure 2E, others not shown). Examining the DNA contents of total cell populations before and 6 hours after treatments demonstrated that the differences seen between these GFP(+) populations were not due to overt differences in cell cycle progression within the assay period (see below).

CEM cells exhibit an NF- κ B-dependent G₂/M cell cycle arrest. Previous studies linking NF- κ B activation with anti-apoptotic activities relied primarily on either (i) increased apoptotic responses when NF- κ B activation was prevented by the expression of a super-repressor I κ B α mutant (20, 42, 43), knock-out of p65 (14, 15), or knock-out of IKK components (16, 17), or (ii) increased resistance to apoptosis when cells were made to overexpress rel members of the NF- κ B protein (44, 45). To our knowledge, there have not been studies that specifically examined the phenotypes of NF- κ B-activated cell populations without prior manipulations of the NF- κ B activation potentials. Thus, we treated CEM κ B cells with IR and FACS sorted them based on the expression of GFP at different time points to examine the behavior of "GFP(+)" and "GFP(-)" populations. While both populations accumulated efficiently at the G₂/M cell cycle phase within 24 hours of exposure (Figure 3A), high percentages of GFP(+) cells remained in this cell cycle phase for up to 72 hours with little apoptosis. By 96 hours, some of these GFP(+) cells had re-entered the cell cycle as detected by the emergence of G₁ and S-phase cells. In contrast, GFP(-) cells failed to remain in the G₂/M cell cycle phase for a prolonged period of time and underwent nearly complete apoptosis within 96 hours. Importantly, these effects were not due to the expression of GFP per se, since GFP(+) cells following exposure to TNF α did not display the similar phenotype (Supplemental Figure 1).

To distinguish whether the maintenance of G₂/M arrest seen in GFP(+) cell populations was due to NF- κ B activation or to some associated parallel events, we expressed the super-repressor I κ B α mutant (S32/36A-I κ B α) in CEM cells and isolated stable clones that failed to efficiently activate NF- κ B when stimulated with IR and VP16 (Figure 3B and others not shown). Figure 3B shows NF- κ B activity in three different clones. CEM-S32/36A-23 displayed the greatest inhibition of NF- κ B activity so this clone was utilized in further experiments. When these cells were stimulated with IR, cell populations displaying prolonged G₂/M arrest were no longer observed and correspondingly increased apoptosis was observed (Figure 3C). These results indicated that the activation of NF- κ B was necessary to maintain G₂/M arrest and protect CEM cells from apoptosis.

P21^{waf1/cip1} is induced in an NF- κ B dependent manner following IR exposure primarily in S/G₂ cell cycle phases. Since NF- κ B has been found to induce antiapoptotic genes (4-8), it is possible that induction of such a gene could represent the mechanism for the maintenance of G₂/M arrest by directly preventing apoptotic cell death of G₂/M arrested cells. Alternatively, it is possible that NF- κ B induces a gene product that directly modulates G₂/M cell cycle arrest. We therefore screened the expression of several antiapoptotic genes and cell cycle regulators following

exposure of CEM and CEM S32/36A cells to IR by ribonuclease protection assay (RPA). We found that p21^{waf1/cip1}, an inhibitor of cyclin-dependent kinases (CDK), was specifically induced in CEM, but not in CEM-S32/36A cells (Figure 4A). In contrast, the antiapoptotic Bcl-XL gene was not induced (Figure 4A). Since p21^{waf1/cip1} is best characterized for its induction by p53 tumor suppressor in G₁ to cause a G₁ cell cycle arrest, we wondered how NF- κ B-dependent induction of p21^{waf1/cip1} was associated with the observed G₂/M cell cycle arrest. This CDK inhibitor has been described to have a role in the maintenance of G₂/M arrest (46). Thus, we considered the possibility that NF- κ B induced p21^{waf1/cip1} in cell cycle phases other than G₁ to regulate G₂/M cell cycle arrest, even though NF- κ B was activated in each phase of the cell cycle, including the G₁ phase. To test this notion directly, we sorted G₁ cell populations by FACS away from cells in S/G₂ phases based on the DNA content after 6 hours following IR exposure. These cells were then analyzed for the expression of p21^{waf1/cip1} mRNA by RPA analysis. Surprisingly, the expression of p21^{waf1/cip1} mRNA was concentrated in the S-G₂/M population compared to the G₁ cells (Figure 4B). It was also not induced in CEM-S32/36A cells, confirming NF- κ B dependence. Quantitative real-time PCR demonstrated that the NF- κ B-dependent induction of p21^{waf1/cip1} mRNA was also seen with another DNA damaging agent, VP16 (Figure 4C). Similar to the results seen with IR, the expression of Bcl-XL was not induced in these cells even with VP16 treatment (Figure 4C). Western blotting further confirmed that p21^{waf1/cip1} protein was indeed induced in an NF- κ B dependent fashion by both IR and VP16 as seen by the lack of p21^{waf1/cip1} protein induction in two different clones expressing the S32/36A super-repressor I κ B α mutant (Figure 4D, and data not shown). Moreover, NF- κ B-dependent induction of p21^{waf1/cip1} was not limited to the CEM cell system and was also seen in p53-mutant MDA-MB-231 human breast cancer cells (Figure 4E and Supplemental Figure 2). Similar to CEM cells, these breast cancer cells also displayed the G₂/M checkpoint maintenance phenotype upon IR exposure, which was reduced when NF- κ B activation was abrogated (Figure 4F).

NF- κ B induction of p21^{waf1/cip1} contributes to G₂/M checkpoint maintenance. To directly test whether NF- κ B induction of p21^{waf1/cip1} was critical to regulate the G₂/M checkpoint maintenance in CEM and MDA-MB-231 cells, we selectively reduced p21^{waf1/cip1} induction by the use of stable RNA interference. A p21^{waf1/cip1} specific RNA interference pSilencer vector was cotransfected with a puromycin expression vector, and puromycin-resistant clones displaying reduced accumulation of p21^{waf1/cip1} following IR treatment were isolated (Figure 5A and 5C). Both CEM and MDA-MB-231 cells showed a reduction in G₂/M checkpoint maintenance and an increase in apoptosis when p21^{waf1/cip1} expression was suppressed (Figures 5B and 5D). The cell cycle distribution of p21^{waf1/cip1}-silenced MDA-MB-231 cells was nearly identical to that seen with those expressing S32/36A-I κ B α (compare Figures 4F and 5D). The effect of G₂/M checkpoint maintenance was modest in CEM cells, possibly due to incomplete silencing efficiency (Figure 5A). These observations demonstrated that NF- κ B dependent p21^{waf1/cip1} induction indeed contributed to G₂/M checkpoint maintenance and survival of these p53-mutant human cancer cell lines.

DISCUSSION

The role of NF- κ B as an apoptosis regulator provided a conceptual framework in which manipulation of NF- κ B activity may serve as a drug target in different pathological settings, including malignancy (3). Currently, there are a number of anti-NF- κ B drugs being developed, primarily targeting the proteasome or IKK activity (47). A common experimental approach to demonstrate the role of NF- κ B in cell survival employs the expression of super-repressor I κ B α , or the use of NF- κ B-deficient cells, such as p65 knockout mouse embryonic fibroblasts, to specifically attenuate NF- κ B activity. In this study, we developed an NF- κ B reporter cell system to positively examine the behavior of NF- κ B activated cells without prior manipulation of NF- κ B activation potential. This system permitted the analysis of populational variations among drug-exposed cells with respect to NF- κ B activation, cell cycle regulation, and survival differences.

We found that activation of NF- κ B occurred in different cell cycle phases following stimulation with the DNA damaging agents, IR and VP16, with no overt bias toward a specific cell cycle phase. Similar observations were also made with TNF α stimulation. In contrast, in accordance with previous findings (20, 21), activation by CPT was concentrated in the S-phase of the cell cycle. These findings provided some conceptual implications regarding the role of NF- κ B in different cell cycle phases. In the case of CPT, the data implied that NF- κ B target genes are only induced in the S or later cell cycle phases depending on the kinetics of NF- κ B-dependent target gene expression and duration of NF- κ B activation. In contrast, IR and VP16 treatments provide the opportunity for NF- κ B to induce its target genes in different cell cycle phases, including the G₁ phase, which may be the predominant cell cycle phase in tumor cells in vivo. It was, however, unclear how different NF- κ B target genes were regulated in different cell cycle phases. Our study demonstrated that the existence of genes that are regulated by NF- κ B in a cell cycle-phase selective manner upon genotoxic stress insult. We found that p21^{waf1/cip1} was induced preferentially in cells enriched for S and G₂/M populations. In this case, the coupling of p21^{waf1/cip1} gene expression to specific cell cycle phase (S and G₂, but not in G₁) was apparently critical, since these cells arrested in G₂/M cell cycle phases to presumably prolong the opportunity to repair damaged DNA and eventual cell cycle re-entry in the face of DNA damage induction. Thus, these positive selection studies revealed a previously unrecognized G₂/M role for NF- κ B to promote cancer cell survival.

How does p21^{waf1/cip1} manifest this G₂/M arrest phenotype? P21^{waf1/cip1} is best characterized as a transcriptional target of p53 to act as a cell cycle inhibitor. Thus, in cells with a functional p53 tumor suppressor protein, this pathway causes cell cycle arrest primarily in the G₁ phase of the cell cycle. P21^{waf1/cip1} is capable of interacting with all of the Cdk complexes (48) and studies have also demonstrated that p53 can regulate the expression of p21^{waf1/cip1} to block cells in the G₂ phase of the cell cycle (49). However, p53 is among the most frequently inactivated tumor suppressor genes in human cancer, and this inactivation is associated with increased resistance to anticancer treatments (22-25). Under p53-defective conditions, p21^{waf1/cip1} can still be induced by other molecular pathways.

Studies have revealed that treatment of certain cell types with anti-cancer agents leads to an accumulation of p21^{waf1/cip1} protein levels and a G₂/M arrest (28, 46, 50,

51). Introduction of nonfunctional p21^{waf1/cip1} or a p21^{waf1/cip1} antisense oligonucleotide diminished the G₂/M arrest phenotype, similar to what we found with RNA interference studies in the present study. Also, studies with esophageal squamous cell carcinoma lines have shown that IR induced p21^{waf1/cip1} and a G₂ cell cycle arrest that could also be diminished when p21^{waf1/cip1} expression was blocked by the introduction of p21^{waf1/cip1} antisense oligonucleotides (51). Besides inhibiting CDK activity directly, interaction of p21^{waf1/cip1} with proliferating cell nuclear antigen was found to be critical for causing a G₂ cell cycle arrest in the p53-deficient DLD1 colon cancer cells (46). The results from our current study suggest the possibility that NF- κ B could play a role in p21^{waf1/cip1} induction in these different p53-defective cancer cases. Moreover, the induction of p21^{waf1/cip1} transcription might have also been linked to specific cell cycle phases (such as S and G₂) to coordinate G₂/M arrest. Thus, NF- κ B activation by genotoxic agents in p53-defective cancer cells may be coupled to G₂/M arrest regulation in a considerable number of human malignancies enhancing cancer resistance.

P21^{waf1/cip1} has also been implicated as a negative regulator of apoptosis in many systems (52). Disruption of p21^{waf1/cip1} following DNA damage switches the cellular outcome from cytostatic to apoptosis. During DNA damage-induced apoptosis, p21 is cleaved by caspase 3 thereby releasing cells from the cell cycle block and increasing apoptosis in A549 human lung cancer cells (53). Following daunorubicin treatment in HCT116 colon cancer cells, the transcription factor myc is recruited to the p21^{waf1/cip1} promoter by the DNA-binding protein Miz-1 thereby blocking p53 from activating p21^{waf1/cip1} transcription (54).

Finally, p21^{waf1/cip1} has been implicated in enhancing NF- κ B dependent gene expression through the modulation of p300 coactivator activity (55). Thus, induction of p21^{waf1/cip1} by NF- κ B may create positive feedback stimulation of NF- κ B dependent transcription in these cancer cells, further promoting their survival. Furthermore, HCT116 colon cancer cells stimulated with daunomycin induced NF- κ B binding to a putative κ B binding site in the p21^{waf1/cip1} promoter (56), even though the phenotype associated with this cell system was not determined. NF- κ B has been shown to induce MDM2 to reduce the expression of p53 in mouse embryonic fibroblasts and enhance their survival (57). It is also possible that under such condition, NF- κ B may also induce p21^{waf1/cip1} to promote G₂/M checkpoint maintenance. Moreover, NF- κ B activation by DNA damaging agents appeared to be highly variable with the presence of different levels of cell populations that did not induce NF- κ B-dependent GFP expression. Further dissection of the mechanisms involved in both populational variation of the NF- κ B response and cell cycle-coupled induction of NF- κ B target genes, including p21^{waf1/cip1}, may provide further insight into understanding cell survival mechanisms provided by this ubiquitous transcription factor.

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FIGURE LEGENDS

Figure 1. DSB-inducing agents activate NF- κ B in CEM human T cells. (A) CEM cells were exposed to various doses of IR (1-40 Gy) and cells were collected 3 hours later. Total cell extracts were analyzed by EMSA using an Ig κ - κ B probe. The lower panel shows an EMSA using an Oct-1 probe for a loading control. (B) CEM cells were exposed to 10 Gy of IR. Cell samples were terminated at indicated time points, and cell extracts were analyzed by EMSA as above (upper panel) or Western blotting with I κ B α antibody (lower panel). (C) CEM cells were treated with various genotoxic agents (10 μ M CPT, 20 μ M TPT, 10 μ M VP16, 25 μ M doxorubicin, or by 20 Gy IR) for 3 hours or TNF α (10 ng/ml) for 20 min. NF- κ B and Oct-1 binding activities were determined by EMSA as described above. (D) CEM cells were transiently transfected with the 3 \times κ B-Luc reporter plasmid. At 48 hours after transfection, cells were treated with TNF α , CPT, Dox, VP16 or IR at the same doses as used in (C) for 6 hours. Cell extracts were analyzed for luciferase activity and standardized to total protein. Error

bars are SD of data obtained from three independent experiments.

Figure 2. NF- κ B activation by CPT but not IR and VP16, is cell cycle-dependent.

(A) CEM κ B cells were treated with 10 ng/ml TNF α or 10 μ M CPT for 24 hours. NF- κ B activation was visualized under fluorescein-aided fluorescent microscopy (right panels) or phase-contrast microscopy (left panels). (B) CEM κ B cells were treated for 6 hours with 10 ng/ml TNF α (upper panel) or 10 μ M CPT (lower panel). NF- κ B activation was determined by measuring GFP fluorescence on a flow cytometer. (C) CEM κ B cells were treated with 10 ng/ml TNF α for 24 hours. After treatment, GFP(+) cells were sorted from GFP(-) cells by flow cytometry. Total cell extracts were analyzed by EMSA using an Ig κ - κ B probe. (C) CEM κ B cells were treated for 6 hours with 10 ng/ml TNF α (upper panel) or 10 μ M CPT (lower panel). NF- κ B activation was determined by measuring GFP fluorescence on a flow cytometer. (D) CEM κ B cells were treated with 10 ng/ml TNF α or 10 μ M CPT for six hours. After treatment, GFP(+) cells were sorted from GFP(-) cells by flow cytometry. Cell cycle profiles were examined by PI staining and analyzed with the Cellquest and Modfit software. (E). CEM κ B cells were treated with 10 μ M VP16 for six hours and GFP(+) cells were sorted from GFP(-) cells by flow cytometry and analyzed as in (D). (F) CEM cells were pretreated with 25 μ M aphidicolin for 30 min (denoted "+" and "-") followed by a 20 minute exposure to 10 ng/ml TNF α , or a 3 hour exposure to 10 μ M CPT, 10 μ M VP16, 25 μ M Dox, or 20 Gy IR. Cell extracts were analyzed for NF- κ B (upper panel) or Oct-1 (lower panel) by EMSA.

Figure 3. NF- κ B-activated cells display a prolonged G₂/M cell cycle arrest followed by cell cycle re-entry.

(A) CEM κ B cells were exposed to 10 Gy of IR and allowed to repair for the times indicated. At each timepoint, CEM κ B GFP(+) cells were sorted from GFP(-) cells using the FACS Vantage cell sorter. Cells were immediately fixed with 70% ETOH, followed by cell cycle analysis by PI staining and modeling as in Figure 2C. The cell cycle profiles for the total, GFP(+), and GFP(-) cell populations at different timepoints are indicated. (B) Different CEM-S32/36A clones were irradiated with a dose of 20 Gy for three hours and monitored for their ability to activate NF- κ B. The corresponding I κ B α protein levels were determined by Western blot analysis (* denotes the exogenous superrepressor I κ B α). (C) CEM and CEM-S32/36A-23 cells were irradiated with a dose of 20 Gy and allowed to recover for the times indicated. Cell cycle profiles were determined as above.

Figure 4. Induction of p21^{waf1/cip1} by NF- κ B in S/G₂ phases results in the maintenance of a G₂/M cell cycle arrest in CEM and MDA-MB-231 cells.

(A) CEM κ B and CEM-S32/36A-23 cells were irradiated at a dose of 20 Gy. After six hours, total RNA was analyzed by ribonuclease protection assays using the hStress-1 template set. Only the sections of the films corresponding to the genes indicated are shown. (B) CEM κ B and CEM-S32/36A-23 cells were irradiated at a dose of 20 Gy. After 5.5 hours of incubation, cells were labeled with Hoechst dye for 30 minutes before sorting the G₁ cells from the S/G₂-enriched cells on the FACS Vantage cell sorter. Total RNA was prepared and ribonuclease protection assays were performed as above. (C) CEM cells were exposed to 10 μ M VP16 for six hours followed by quantification of

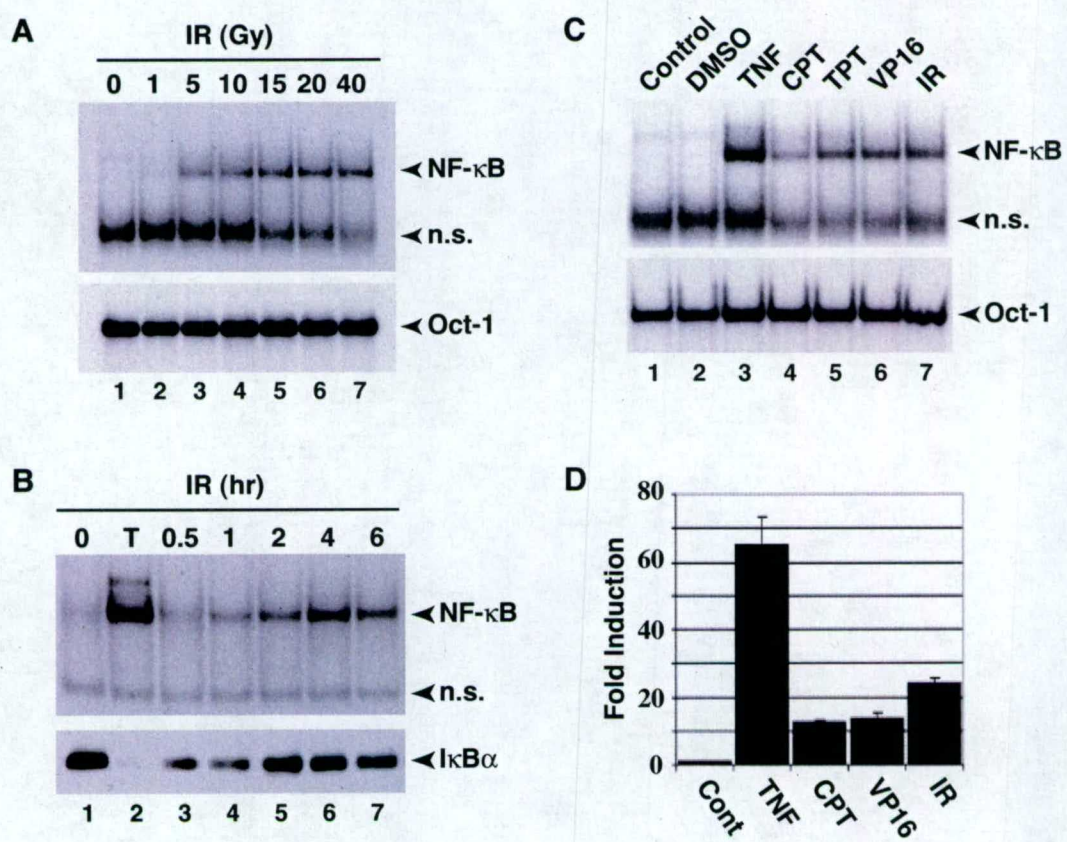
p21^{waf1/cip1} and Bcl-X_L RNA expression levels using real time RT-PCR. (D) P21^{waf1/cip1} protein induction was determined by IP-Western in both CEM and CEM-S32/36A clones after stimulation by 10 ng/ml TNF α (T) or 10 μ M VP16 (V) for 3 hours. (E) MDA-MB-231 and MDA-S32/36A-9 cells were treated with 20 Gy of IR for up to 72 hours. Total cell extracts were prepared and Western analysis was done using the corresponding antibodies as shown. (F) MDA-MB-231 and MDA-S32/36A-9 cells were exposed to 20 Gy of IR for up to 72 hours. Cells were prepared as in Figure 3A and cell cycle profiles were determined at every 24 hour interval.

Figure 5. NF- κ B induction of p21^{waf1/cip1} contributes to G₂/M checkpoint maintenance. (A) A pSilencer p21^{waf1/cip1} stable knockdown clone (clone 9) and a control pSilencer scramble clone were tested for their ability to reduce p21^{waf1/cip1} protein expression following 20 Gy of IR for six hours as above. (B) Statistical analysis of 3 independent experiments showing that pSilencer-Scr cells depict a statistically significant sigmoid curve, indicating that these cells are capable of maintaining a G₂/M arrest. In contrast, pSilencer-p21-9 cells show a statistically significant quadratic polynomial curve, indicating the lack of G₂/M maintenance at the 48 hours time point. (C) MDA-pSi-p21 cells were tested for their p21^{waf1/cip1} protein expression compared to MDA-pSi-Scr control by Western blot analysis following treatment with 20 Gy of IR at the indicated times. (D) MDA-pSi-Scr and MDA-pSi-p21 cells were treated with 20 Gy of IR. Cells were fixed at the indicated time points and cell cycle profiles were determined as in Figure 3A.

Supplemental Figure 1. NF- κ B-activated cells treated with TNF α do not display a G₂/M arrest. CEM κ B cells were exposed to 10 ng/ml TNF α and allowed to recover for the times indicated. At each timepoint, CEM κ B GFP(+) cells were sorted from GFP(-) cells using the FACSVantage cell sorter. Cells were immediately fixed with 70% ETOH, followed by cell cycle analysis by PI staining and modeling as in Figure 2C. The cell cycle profiles for the total, GFP(+), and GFP(-) cell populations at different timepoints are indicated.

Supplemental Figure 2. MDA-S32/36A clones were unable to induce NF- κ B activation following TNF α exposure. MDA-S32/36A clones were tested for their ability to induce NF- κ B following 15 min. of TNF α (10 ng/ml) stimulation by EMSA (upper panel), Parallel samples were examined for I κ B α protein levels by Western blot analysis (lower panel).

Figure 1



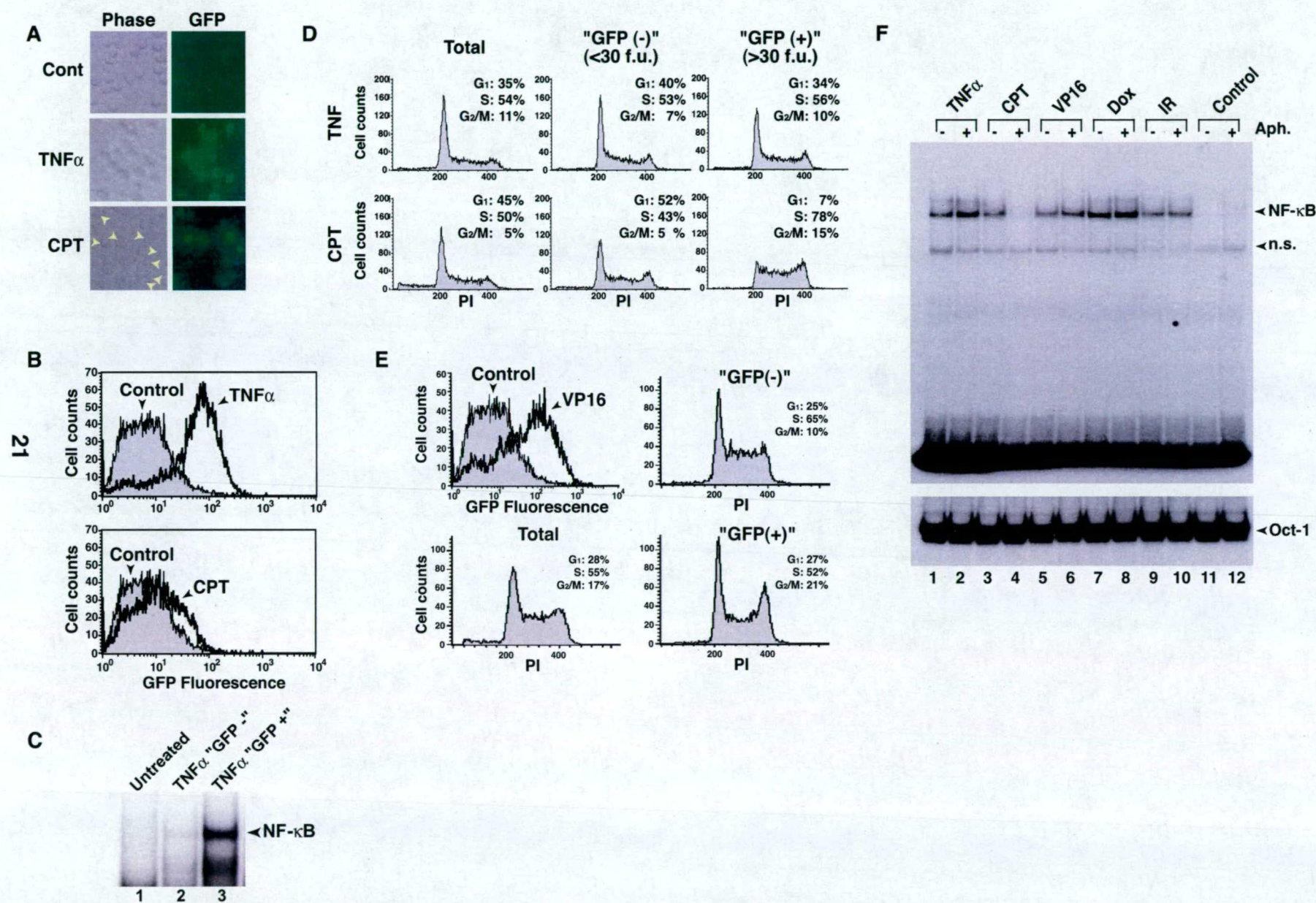
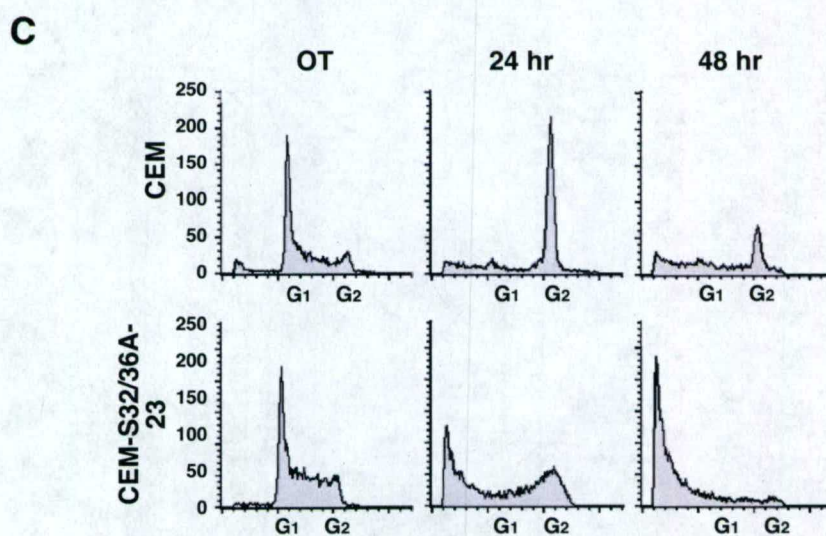
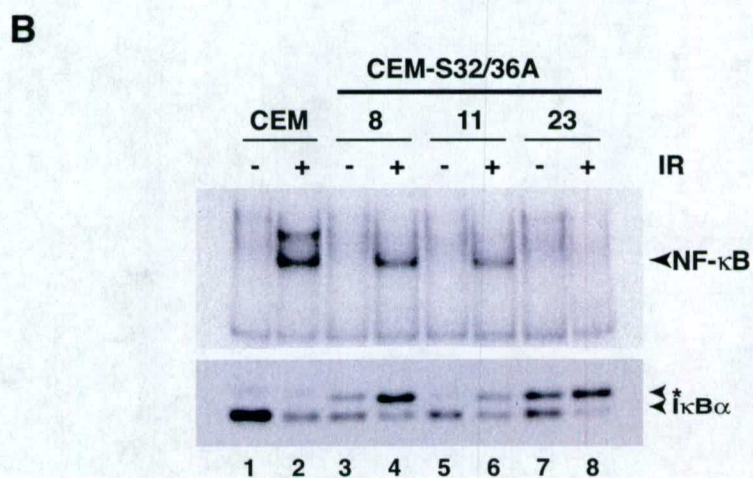
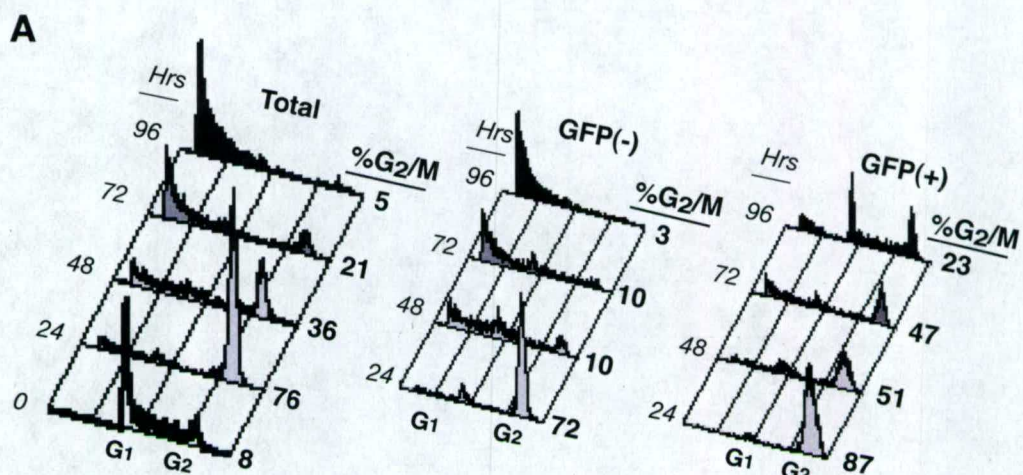


Figure 3



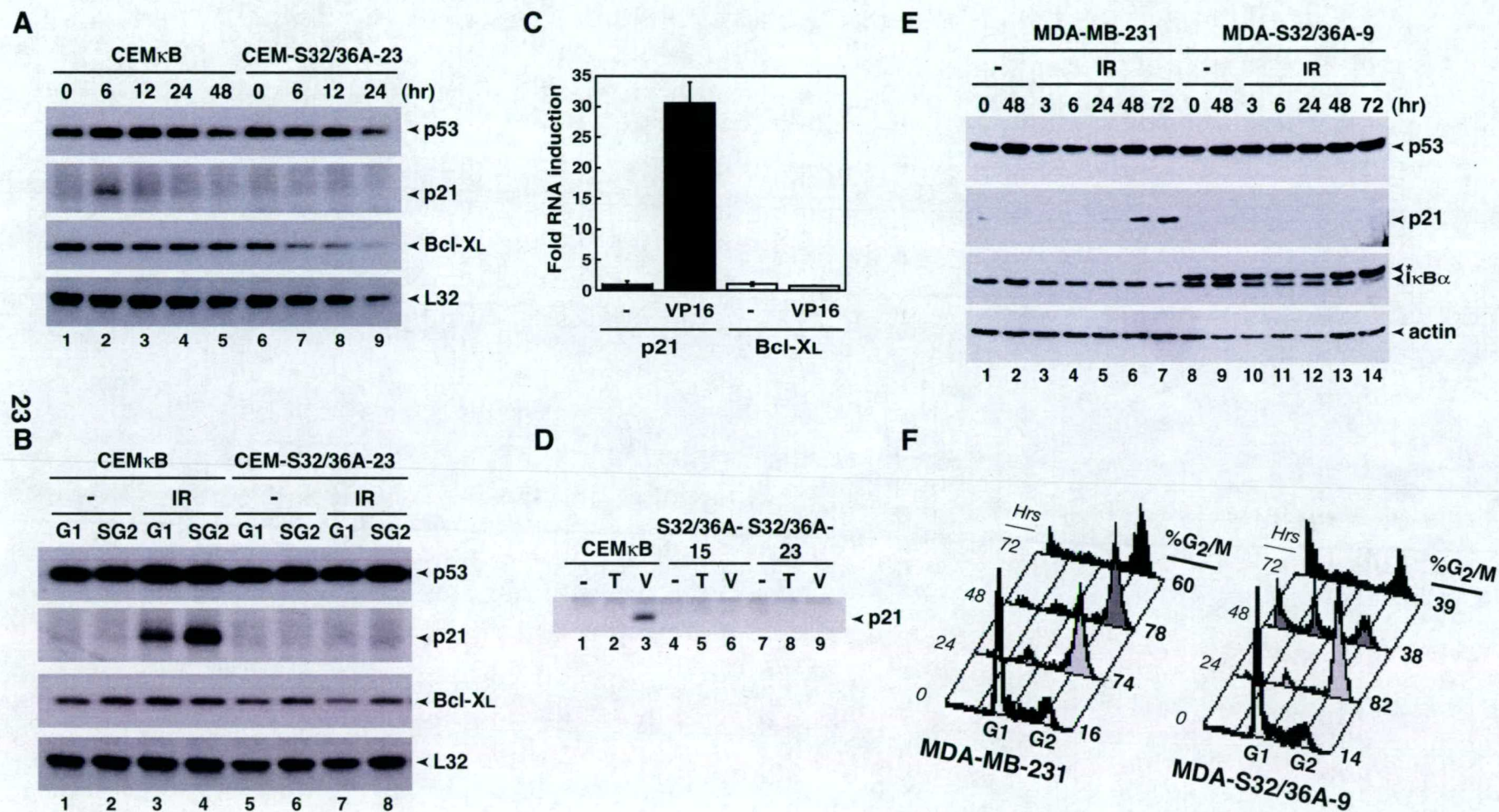
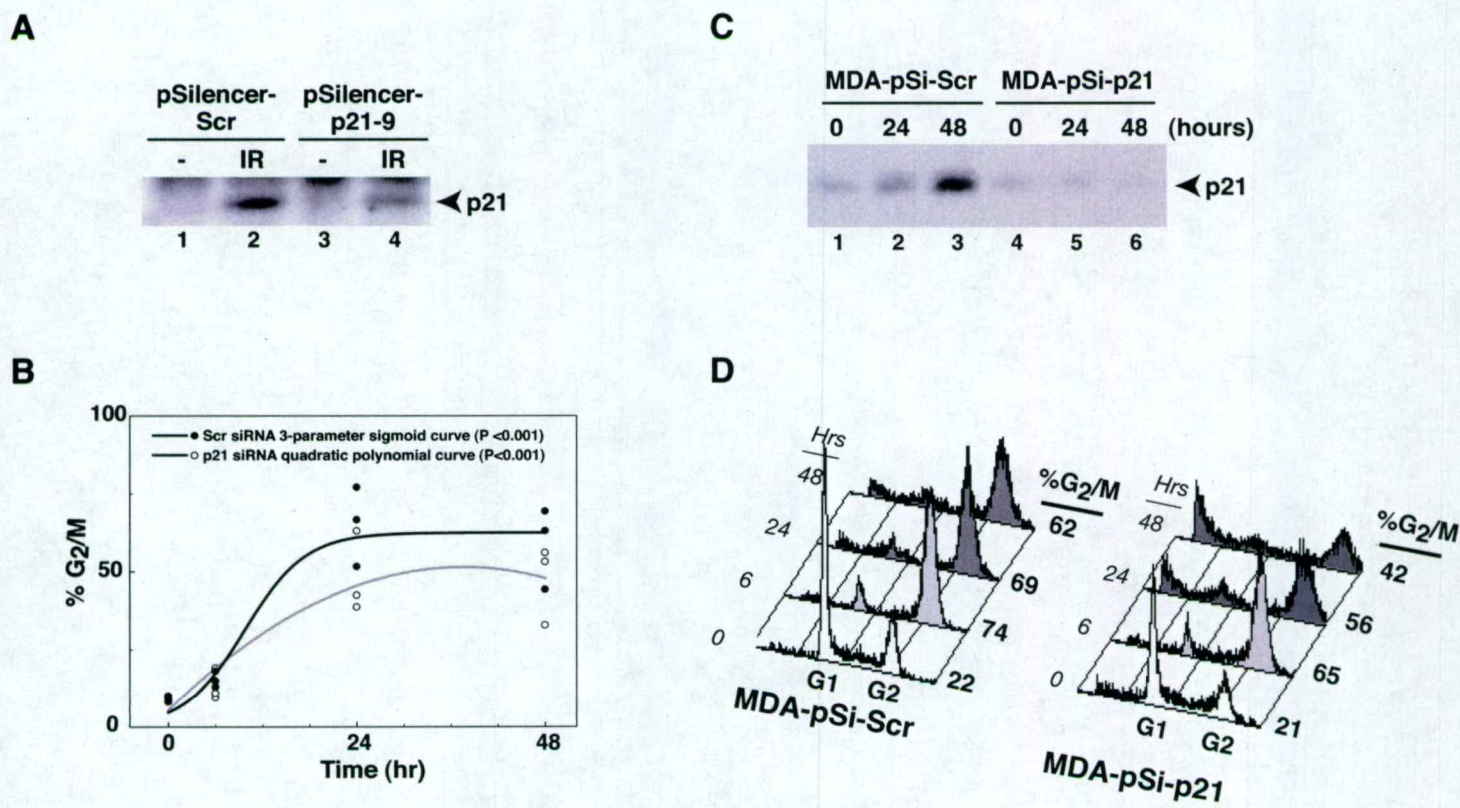
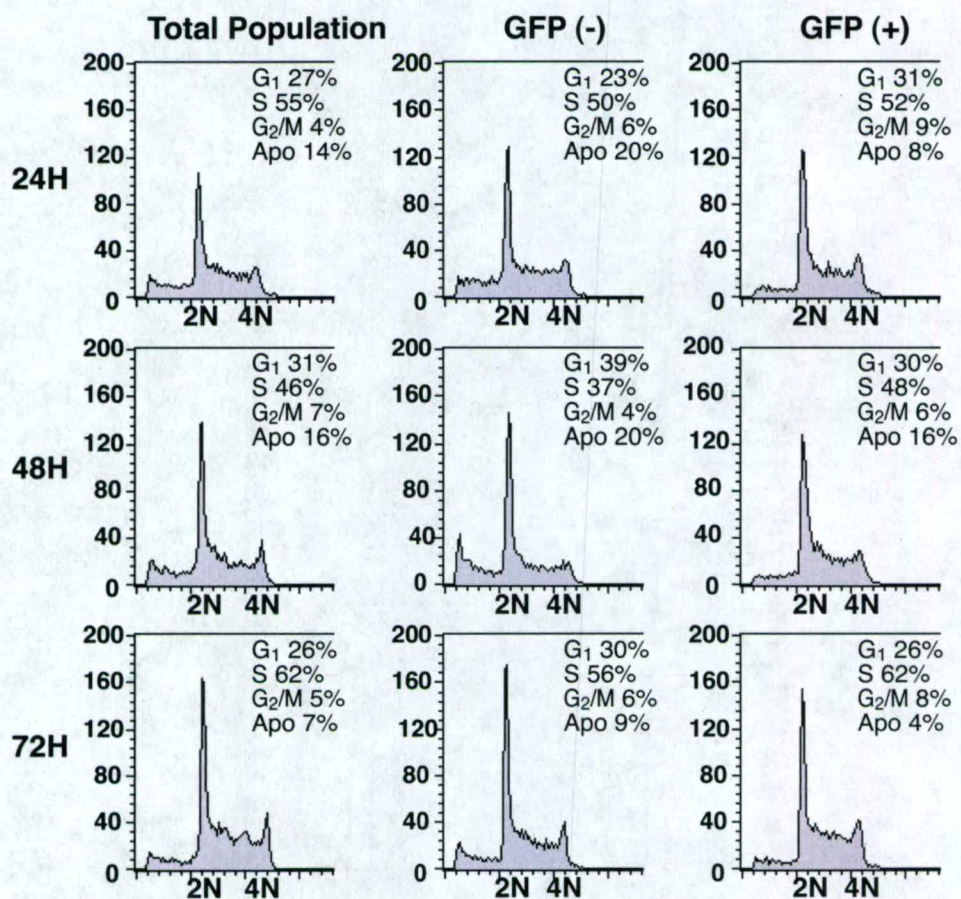


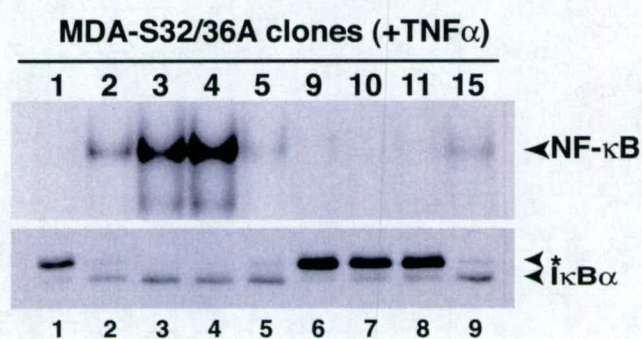
Figure 5



Supplement Figure 1



Supplemental Figure 2



III. Key Research Accomplishments and Conclusions

1. NF- κ B was able to bind to a putative NF- κ B consensus site in the p21^{WAF1/cip1} promoter.
2. A clonal MDA breast cancer cell line and a CEM T leukemic cell line harboring the RNA interference vector specifically knocking down p21.
3. Cell cycle profiles of MDA versus MDA pSilencer p21 knockdown cell lines following treatment with 20 Gy of irradiation indicated that MDA cells display a more abundant and prolonged G₂/M arrest than MDA pSilencer p21 cells, showing a dependence on p21 for the maintenance of the G₂/M arrest.

III. Reportable Outcomes

1. Sequential modification of NEMO/IKK γ by SUMO-1 and ubiquitin mediates NF- κ B activation by genotoxic stress. Huang TT, **Wuerzberger-Davis SM**, Wu ZH, Miyamoto S, Cell. 2003 Nov 26;115(5):565-76.
2. Enhanced cancer cell survival by NF- κ B-dependent p21^{waf1/cip1} induction. **Shelly M. Wuerzberger-Davis**, Pei-Yun Chang, and Shigeki Miyamoto (in submission).

IV. Shelly M. Wuerzberger-Davis completed her doctorate and graduated in Dec. 2004.

V. Conclusions

Task 1: We have shown that there is a NF- κ B dependent G₂/M cell cycle arrest in MDA human breast cancer cells. We are currently working on determining the radioresistance of both cell lines, MDA and MDAS32/36A by colony forming assays along with MDA-pSil-Scr and MDA-pSil-p21 in order to see if there is a growth advantage in cells that maintain a prolonged G₂/M arrest.

Task 2: We have shown that p21^{WAF1/cip1} is a target of NF- κ B through promoter analysis and electrophoretic mobility shift assays. Through the use of stable RNA interference, we were able to show that loss of p21^{WAF1/cip1} does correlate with a reduction in the G₂/M cell cycle arrest as does loss of NF- κ B activation. However, this reduction is only partial. One reason may be due to the fact that the knockdown produced by the pSilencer vectors is not complete, moreover, we do not believe that p21^{WAF1/cip1} is the only gene involved. We are currently preparing RNA to due affimetrix chip analysis (subtask (c)). We will be looking for differences in gene induction following VP16 treatment between parental cells and those transfected with the super-repressor S32/

36A-I κ B α construct.

Breast cancer is one of the most common forms of cancer among women. Efficacious treatment of breast cancer patients is one of the most urgent goals. While current treatment regimens are effective, some cancer cells escape the death inducing effects of anticancer treatments. Activation of the transcription factor NF- κ B has been implicated as one mechanism contributing to cancer resistance. However, mechanisms by which NF- κ B contributes to cancer resistance are not well understood. We have obtained evidence that NF- κ B activation by irradiation can cause a prolonged G₂/M cell cycle arrest in the human breast cancer cell line, MDA-MB-231. The elucidation of the mechanisms involved in cell cycle regulation by NF- κ B after DNA damage will help us not only better understand how to increase the efficacy of breast tumor treatments, but also lead to the identification of a novel gene, such as p21^{waf1/cip1}, whose protein product may prove to be a better target for breast cancer therapy.